11. Guo WJ, Datta S, Band V, Dimri GP. Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. Mol Biol Cell 2007;18: 536–46.
12. Zheng SL, Sun J, Wiklund F, Smith S, Stattin P, Li G, Adami HO, Hsu FC, Zhu Y, Bälter K, Kader AK, Turner AR, Liu W, Bleecker ER, et al. Cumulative association of five genetic variants with prostate cancer. N Engl J Med 2008;358:910–19.
13. Sun J, Chang BL, Isaacs SD, Wiley KE, Wiklund F, Stattin P, Duggan D, Carpten JD, Trock BJ, Partin AW, Walsh PC, Grönberg H, et al. Cumulative effect of five genetic variants on prostate cancer risk in multiple study populations. Prostate 2008;68:1257–62.
14. Sobin LH, Wittekind C, eds. International Union against Cancer: Prostate. In TNM classification of malignant tumors. New York: Wiley-Liss, 2002. p. 184–7.
15. Gleason DF. Histological grading and clinical staging of prostate carcinoma. In: Tannenbaum M: Urologic pathology. New York: Wiley-Liss, Inc., 1977:171–97.
16. Fleming ID. Prostate Cancer: In AJCC Cancer Staging Manual 6th edition. New York: Springer, 2002:309–16.
17. Tsuchiya N, Wang L, Suzuki H, Segawa T, Fukuda H, Narita S, Shimbo M, Kamoto T, Mitsumori K, Ichikawa T, Ogawa O, Nakamura A, Habuchi T. Impact of IGF-I and CYP19 gene polymorphisms on the survival of patients with metastatic prostate cancer. J Clin Oncol 2006;24:1982–9.
18. Nogawa M, Yuasa T, Kimura S, Tanaka M, Kuroda J, Sato K, Yokota A, Segawa H, Toda Y, Kageyama S, Yoshiki T, Okada Y, Maekawa T. Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. J Clin Invest 2005;115:978–85.
19. Mishra PJ, Humeniuk R, Mishra PJ, Longo-Sorbello GS, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. Proc Natl Acad Sci USA 2007;104:13513–18.
20. Yu Z, Li Z, Jolicoeur N, Zhang L, Fortin Y, Wang E, Wu M, Shen SH. Aberrant allele frequencies of the SNPs located in microRNA target sites are potentially associated with human cancers. Nucleic Acids Res 2007;35:4535–41.
21. Landi D, Gemignani F, Naccarati A, Pardini B, Vodicka P, Vodickova L, Novotny J, Försti A, Hemminki K, Canzian F, Landi S. Polymorphisms within micro-RNA-binding sites and risk of sporadic colorectal cancer. Carcinogenesis 2008;29:579–84.
22. Zeng Y, Wagner EJ, Cullen BR. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol Cell 2002;9:1327–33.
23. Zeng Y, Yi R, Cullen BR. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc Natl Acad Sci USA 2003;100:9779–84.
24. Quinn DI, Henshall SM, Sutherland RL. Molecular markers of prostate cancer outcome. Eur J Cancer 2005;41:858–87.

Original Paper

Urologia Internationalis

Urol Int 2009;83:141–145
DOI: 10.1159/000230013

Received: April 22, 2008
Accepted after revision: July 1, 2008

Cystoprostatectomy as a Treatment of Prostate Cancer Involving the Bladder Neck

Teruaki Kumazawa[a] Norihiko Tsuchiya[a] Mitsuru Saito[a] Takamitsu Inoue[a] Shintaro Narita[a] Youhei Horikawa[a] Takeshi Yuasa[a] Shigeru Satoh[a] Tetsuro Kato[a] Hiroshi Nanjyo[b] Tomonori Habuchi[a]

[a]Department of Urology, Akita University School of Medicine, and [b]Clinical Pathology, Akita University Hospital, Akita, Japan

Key Words
Bladder neck invasion · Cystoprostatectomy · Prostate cancer

Abstract
Objective: We evaluated the clinicopathological findings and short- and long-term outcomes of prostate cancer (PCa) patients with bladder neck invasion who underwent cystoprostatectomy. ***Patients and Methods:*** Between 1989 and 2005, we performed 17 cystoprostatectomies for PCa patients having bladder neck invasion without distant visceral or distant lymph node metastasis. Of the 17 patients, 11 were treated with neoadjuvant hormone therapy and all patients were treated with adjuvant hormone therapy immediately after surgery. ***Results:*** All 7 patients in whom pelvic lymph node swelling was identified by preoperative imaging studies had pathological lymph node metastasis. Of the 10 patients judged as cN0 preoperatively, 7 (70.0%) had lymph node metastasis. Although local recurrence was found in 2 (11.8%) patients, no additional urinary diversion or inconvenient urinary symptoms due to PCa progression were observed in any patients. The 5-year prostate-specific antigen recurrence-free survival rate was 62.2%. Cause-specific survival at 5 years after surgery was 87.1%. The 5-year cause-specific survival rate of node-positive patients was 92.3%. ***Conclusion:*** Cystoprostatectomy followed by immediate hormone therapy may be a feasible treatment option to achieve excellent local control for patients with previously untreated PCa, even in the presence of pelvic lymph node metastasis.

Introduction

The ideal treatment for locally advanced prostate cancer (PCa) has long been controversial. According to the European Association of Urology (EAU) guidelines for cT3–4 PCa, the most recommended treatments are radiation, hormone therapy or a combination of both, whereas radical prostatectomy is described as an optional treatment only for selected patients with cT3 [1]. Although those non-surgical treatments are usually applied for patients with locally advanced PCa involving the bladder neck, such conservative treatments often result

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0042–1138/09/0832–0141$26.00/0

Accessible online at:
www.karger.com/uin

Tomonori Habuchi
Department of Urology
1-1-1 Hondo, Akita 010-8543 (Japan)
Tel. +81 18 834 1111, Fax +81 18 836 2619
E-Mail thabuchi@doc.med.akita-u.ac.jp

Table 1. Patients' clinical characteristics of PCa involving the bladder neck

Patients	17
Age, years	64.7 ± 5.8
PSA at diagnosis, ng/ml	35.5 ± 33.0
Preoperative PSA, ng/ml	20.5 ± 26.2
cN status	
N0	10
N1	7
Neoadjuvant	
Yes	11
No	6

Table 2. Pathological findings of cystoprostatectomy

Pathological stage	
T3N0	1 (5.8%)[1]
T3N1	2 (11.8%)[1]
T4N0	2 (11.8%)
T4N1	12 (70.6%)
Gleason score	
8	1 (5.8%)
9	14 (82.4%)
10	2 (11.8%)
Bladder invasion	14 (82.4%)
Seminal vesicle invasion	13 (76.5%)

[1] These patients received neoadjuvant hormone therapy before the operation.

in a refractory state and patients may have to depend on ureteral stents, urethral catheters, or nephrectomy tubes for a long time. Furthermore, such patients may have to undergo multiple invasive procedures such as ureteral catheterization and nephrostomy, which cause a significant decline in quality of life (QOL).

Few studies have evaluated the outcome of cystoprostatectomy as a treatment of PCa with invasion to adjacent organs (cT4) because of a decreased incidence of cT4 disease, and very few institutions perform radical surgery for these patients; however, Leibovici et al. [2] reported the effectiveness of salvage cystoprostatectomy for patients with primary cT4 disease and local recurrence following radiation therapy. They demonstrated that cystoprostatectomy for primary cT4 tumor provided effective and durable palliation of lower urinary tract symptoms (LUTS). Recent data from the Surveillance Epidemiology and End Results (SEER) revealed that patients who underwent radical prostatectomy for cT4 PCa had increased survival compared with patients who received radiation therapy alone or hormone therapy alone and had a survival comparable to that of patients who received combined therapy of radiation and hormone therapy [3]. Furthermore, the study indicated the survival of radical prostatectomy over combined radiation therapy with hormonal therapy for cT4 PCa with lymph node metastases [3]. Among cT4, compared to patients with PCa involving the rectum or pelvic floor muscles, those with bladder neck invasion may be better candidates for cystoprostatectomy to achieve local cancer control and improvement of QOL. In this study, we assessed the impact of cystoprostatectomy on the clinical outcome of patients with cT4 disease, especially with bladder neck invasion.

Patients and Methods

Between 1989 and 2005, we performed 17 cystoprostatectomies for PCa patients having bladder neck invasion without distant visceral or distant lymph node metastasis. Patients' clinical characteristics are shown in table 1. The mean age at surgery was 64.7 years. The mean serum prostate-specific antigen (PSA) level at surgery was 35.5 ng/ml. In all patients, bone scan and abdominal computerized tomography (CT) were used for preoperative staging. All patients were diagnosed with bladder neck invasion of PCa by cystourethroscopy with or without bladder neck biopsy. Neoadjuvant hormone therapy, i.e. surgical or medical castration with LH-RH analogue or complete androgen blockage by adding antiandrogen agents, was performed in 11 (64.7%) patients. Ileal conduit, rectal neobladder, and Kock pouch were constructed for urinary diversion in 7, 9, and 1 patients, respectively [4]. Pathological evaluation, including the Gleason Score (GS), was performed by a single pathologist (H.N.). After surgery, all patients were treated with immediate adjuvant hormone therapy, which included surgical or medical castration, complete androgen blockage, or estramustine phosphate. PSA recurrence was defined when the PSA level had risen to a level >0.4 ng/ml after surgery. If the PSA level did not decrease <0.4 ng/ml, we considered the operation day as recurrence day. The median follow-up period after operation was 89.0 months. Overall survival and cause-specific survival measured from the date of the surgery was estimated by the Kaplan-Meier product limit method.

Results

Pathological findings are shown in table 2. All 7 patients in whom pelvic lymph node swelling was identified by CT scan had pathological lymph node metastases. Of the 10 patients in whom pelvic node swelling was not detected preoperatively, 7 (70.0%) had lymph node metastasis. All 17 patients had high-grade tumors (GS 8–10)

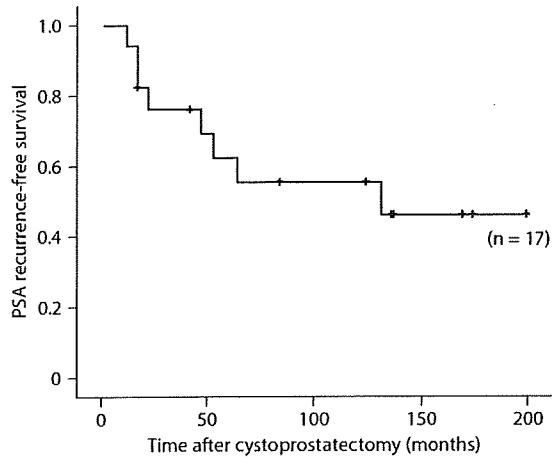


Fig. 1. PSA recurrence-free survival in PCa patients with bladder neck invasion. The projected 5-year PSA recurrence-free survival rate was 62.2%.

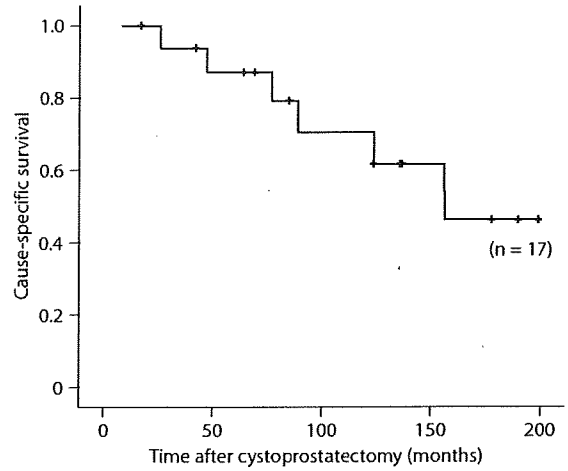


Fig. 2. Cause-specific survival in PCa patients with bladder neck invasion. The actuarial cause-specific survival at 5 years was 87.1%.

and 14 (82.4%) had GS 9 tumors. Cancer invasion into the bladder neck was pathologically demonstrated in 14 patients (82.4%). Seminal vesicle invasion was found in 13 patients (76.5%).

PSA recurrence after surgery was observed in 8 patients (47.1%). The projected 5-year PSA recurrence-free survival rate was 62.2% (fig. 1). Six patients (35.3%) died as a direct result of cancer progression. One patient died of causes not related to PCa with no evidence of biochemical recurrence. The actuarial cause-specific survival at 5 years was 87.1% (fig. 2). The median survival was 156 months.

Of 14 patients with pelvic lymph node metastasis, 6 patients died of PCa. The 5-year survival rate of pN1 patients was 92.3%. The median survival of pN1 patients was 124 months. Although the number of patients was small, there was no significant difference in cause-specific survival between pN1 and pN0 patients (fig. 3).

There were no treatment-related deaths or major perioperative complications (table 3). Of the 17 patients, 2 (11.8%) developed local recurrence but had no subjective symptoms until death. One patient had an ileal conduit diversion 65 months after cystoprostatectomy with rectosigmoidal neobladder construction due to primary rectal cancer. Nine patients whose death related to PCa were not concerned by urinary tract symptoms before death.

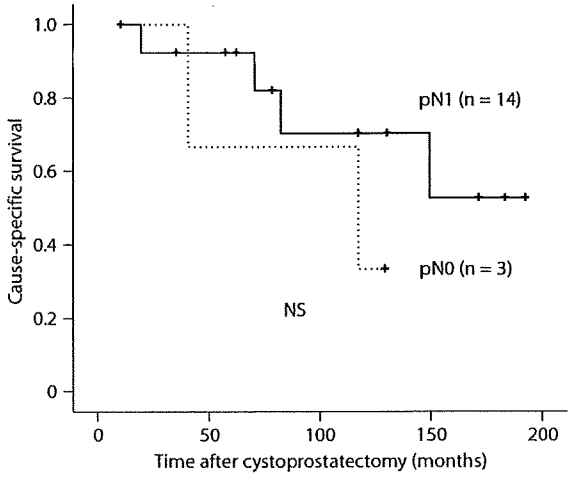


Fig. 3. Cause-specific survival stratified by pN status. There was no significant difference between pN1 and pN0 patients.

Discussion

PCa involving the bladder neck often irritates patients with various LUTS, including micturition pain, dysuria, ischuria, and hematuria, and is recommended to be treat-

Table 3. Perioperative complications

Wound infection	3 (17.6%)
Prolonged intestinal paralysis	6 (35.3%)
Pelvic abscess	1 (5.9%)
Acute pyelonephritis	1 (5.9%)

ed with radiation therapy and/or hormone therapy in most of the current guidelines [1]. However, the refractory state after conservative treatments often worsens the patients' QOL by recurrent LUTS, prolonged urine drainage using a catheter or stent in the urinary tract which sometimes induces complications such as dislodgement, obstruction, bladder spasm, bleeding and infection. Leibovici et al. [2] recently reported the clinical implication of cystoprostatectomy in 17 primary cT4 PCa patients with a mean follow-up period of 25 months and concluded that cystoprostatectomy provides effective and durable palliation in patients with locally advanced PCa. Similarly, in our institution, cystoprostatectomy was performed in PCa patients with bladder neck invasion showing severe LUTS for the purpose of relieving those symptoms. All patients in our study, including patients who developed local recurrence, had no local symptoms or no need for catheters for urinary tract obstruction until death. Although no study has compared QOL after conservative treatment and surgical intervention in PCa patients with bladder neck involvement, our results indicate that cystoprostatectomy may be a treatment option in these patients.

In our relatively long-term follow-up study with a median period of 89.0 months, the 5-year cause-specific survival rate reached 87.1%, although the number of patients was small. Fowler et al. [5, 6] reported that the 5-year survival rate of patients with locally progressive PCa who were treated with hormone therapy alone was 92% with a mean follow-up of 78 months. Bolla et al. [7] compared the results of combined hormone therapy and radiotherapy with those of radiotherapy alone in locally advanced PCa and reported that overall survival at 5 years was 79% in the combination group and 62% in the radiotherapy-alone group. When performing radical cystoprostatectomy for PCa involving the bladder neck, the possibility of overtreatment should be considered; however, all patients with PCa involving the bladder neck had high-grade (GS 9–10) cancer in this study, which is a potent significant risk factors of poor outcome, and a considerable number of these patients are likely to develop disease progression and inconvenient LUTS in a short period of time. Since salvage surgery for recurrent PCa after radiation therapy is known to be expected with a high incidence of morbidity [8–10], cystoprostatectomy as an initial treatment for these patients is suggested to be acceptable from the aspect of both efficacy, morbidity and mortality. On the other hand, of 17 patients, 3 (17.6%) were downstaged to T3 disease, suggesting that radical prostatectomy rather than cystoprostatectomy might have been indicated for those patients. In our study, employing retrospective analysis, it is possible that some bias in patient selection may affect the evaluation of the clinical outcome. Further study should be conducted to elucidate which patients are likely to benefit from cystoprostatectomy in terms of prolongation of survival.

Our study showed that, although patients with PCa involving the bladder neck had frequent lymph node metastasis (82.4%), the 5-year cause-specific survival rate of patients even with lymph node metastasis was 92.3%, which showed no significant difference in survival between pN0 and pN1 patients. Recently, Johnstone et al. [3] reported that radical prostatectomy with or without hormone therapy appeared to have a survival benefit, especially in cT4 PCa patients with positive regional lymph node compared to those with negative lymph node status. Messing et al. [11] reported that immediate hormone therapy after radical prostatectomy and pelvic lymphadenectomy improved 5-year cause-specific survival by about 15% in patients with node-positive PCa, although the study targeted patients with cT2 disease. From the results of these studies and our study, cystoprostatectomy is suggested to be applicable in cT4 PCa patients with node-positive disease. In our study, all the 17 patients received adjuvant hormone therapy immediately after the surgery. However, such adjuvant hormonal therapy might not be required in some patients when an undetectable postoperative PSA level was achieved. In addition, some patients could have benefited from postoperative radiotherapy instead of adjuvant hormonal therapy.

In conclusion, cystoprostatectomy followed by immediate hormone therapy may be a feasible option for selected patients with previously untreated PCa involving the bladder neck because of excellent local control and long-term survival. A further large-scale study with a longer follow-up is warranted to validate extensive surgical management as part of a multidisciplinary approach for locally advanced PCa and to elucidate which patients benefit from such surgical treatment regarding survival and QOL.

References

1 Aus G, Abbou CC, Bolla M, Heidenreich A, Schmid HP, van Poppel H, Wolff J, Zattoni F: EAU Guidelines on Prostate Cancer. Eur Urol 2005;48:546–551.

2 Leibovici D, Kamat AM, Pettaway CA, Pagliaro L, Rosser CJ, Logothetis C, Pisters LL: Cystoprostatectomy for effective palliation of symptomatic bladder invasion by prostate cancer. J Urol 2005;174:2186–2190.

3 Johnstone PA, Ward KC, Goodman M, Assikis V, Petros JA: Radical prostatectomy for clinical T4 prostate cancer. Cancer 2006;106:2603–2609.

4 Kato T, Sato K, Miyazaki H, Sasaki S, Matsuo S, Moriyama M: The uretero-ileoceco-proctostomy (ileocecal rectal bladder): early experiences in 18 patients. J Urol 1993;150:326–331.

5 Fowler JE, Bigler SA, Kolski JM, Yee DT: Early results of a prospective study of hormone therapy for patients with locally advanced prostate carcinoma. Cancer 1997;82:1112–1127.

6 Fowler JE, Bigler SA, White PC, Duncun WL: Hormone therapy for locally advanced prostate cancer. J Urol 2002;168:546–549.

7 Bolla M, Gonzalez D, Warde P, Dubois JB, Mirimanoff RO, Storme G, Bernier J, Kuten A, Stemberg C, Gil T, Collette L, Pierart M: Improved survival in patients with locally advanced prostate cancer treated with radiotherapy and goserelin. N Engl J Med 1997;337:295–300.

8 Shekarriz B, Upadhyay J, Pontes JE: Salvage radical prostatectomy. Urol Clin North Am 2001;28:545–553.

9 Gheiler EL, Tefilli MV, Tiguert R, Friedland MS, Frontera RC, Pontes JE: Predictors for maximal outcome in patients undergoing salvage surgery for radio-recurrent prostate cancer. Urology 1998;51:789–795.

10 Lerner SE, Blute ML, Zincke, H: Critical evaluation salvage surgery for radio-recurrenct/resistant prostate cancer. J Urol 1995;154:1103–1109.

11 Messing EM, Manola J, Yao J, Kiernan M, Crawford D, Wilding G, di'SantAgnese PA, Trump D, Eastern Cooperative Group study EST 3886: Immediate versus deferred androgen deprivation treatment in patients with node-positive prostate cancer after radical prostatectomy and pelvic lymphadenectomy. Lancet Oncol 2006;7:472–479.

BJU INTERNATIONAL

Tumour length of the largest focus predicts prostate-specific antigen-based recurrence after radical prostatectomy in clinically localized prostate cancer

Ryuichi Mizuno, Jun Nakashima, Makio Mukai*, Hajime Okita*, Michio Kosugi, Eiji Kikuchi, Akira Miyajima, Ken Nakagawa, Takashi Ohigashi and Mototsugu Oya

*Departments of Urology and *Diagnostic Pathology, Keio University School of Medicine, Tokyo, Japan*

Accepted for publication 9 February 2009

Study Type – Prognosis (case series)
Level of Evidence 4

OBJECTIVE

To investigate the possible significance of tumour dimensional variables, including maximum tumour diameter (MTD), maximum tumour area (MTA) and total tumour volume (TTV), with standard prognostic factors for predicting prostate-specific antigen (PSA) recurrence after radical prostatectomy (RP).

PATIENTS AND METHODS

Serial whole sections of the prostate from 164 patients who had RP for localized prostate cancer were investigated. Cox proportional hazards regression models were used for univariate and multivariate analyses to test the relationships between biochemical failure and clinicopathological factors, including tumour dimensional variables. The results were analysed retrospectively to develop a prognostic factor-based model for risk stratification.

RESULTS

In the univariate Cox proportional hazard model, pathological T stage, Gleason score, perineural invasion, microvascular invasion, positive surgical margins, MTD, MTA and TTV were significantly associated with biochemical failure. In the multivariate Cox proportional hazard model using a stepwise inclusion of these factors, Gleason score, positive surgical margins and MTD were independent indices in association with biochemical failure. Using the three statistically significant variables, the relative risk of biochemical failure could be calculated.

CONCLUSION

These results imply that MTD is possibly one of the most important prognostic factors for predicting biochemical recurrence after RP. As calculating the MTD on the section a rapid, simple and objective method, it can be used instead of the TTV calculation. The prognostic factor- based risk stratification might help clinicians to predict biochemical failure after RP.

KEYWORDS

prostate cancer, tumour volume, tumour diameter, biochemical recurrence

INTRODUCTION

A standard treatment for patients with clinically localized prostate cancer is radical prostatectomy (RP). It has been reported that recurrent detectable serum PSA levels after RP are a reliable indicator of residual cancer that can provide evidence of tumour recurrence months or years before a clinical determination of treatment failure [1]. Biochemical failure is not uncommon after RP and it is important to characterize the pathological features of the RP specimen that might be used to predict biochemical failure and progression, to predict recurrence and initiate adjuvant therapy. The pathological assessment of RP specimens can identify features, such as Gleason score, margin status, vascular/lymphatic invasion, perineural invasion (PNI) and tumour dimensions. Among these variables, tumour volume is the most commonly used measure of the amount of tumour, which is a well-recognized predictor of biological behaviour in many organs. In the case of prostate cancer, tumour volume has particularly been considered to be a major determinant of the biological behaviour [2,3]. There are several different methods of evaluating the tumour volume in prostate cancer, but accurate measurement is difficult and time-consuming because tumours are often multifocal. Therefore, an alternative method of estimating tumour size is desirable. Among tumour size variables, tumour length or area of the largest focus could be good candidates for fast, easy and objective methods of estimating the amount of tumour [4–7].

We investigated the possible significance of tumour dimensional variables, with standard prognostic factors, for predicting PSA recurrence after RP. Risk stratification is an important issue in cancer treatment because it can facilitate a more accurate prediction of outcome and provide a more homogenous population for the most appropriate therapeutic approach. Therefore in the present study a prognostic factor-based model for risk stratification was developed.

PATIENTS AND METHODS

In all, 164 patients (mean age 65.6 years, range 52–74) were included in the study; patients were excluded when radiotherapy or androgen-deprivation therapy was used before RP. The mean (median) preoperative serum PSA level was 11.5 (8.0) ng/mL. All patients had RP at our institution, and prostate cancer was histologically confirmed before RP by TRUS-guided needle biopsy using a 10-MHz endorectal transducer. Indications for prostate biopsy were an abnormal DRE and/or a serum PSA level of >4.0 ng/mL. Clinical stage was assessed by the DRE, abdominal and pelvic CT, MRI and bone scan, in accordance with the 1997 TNM classification. Of the 164 patients, 114 were considered to have clinical stage T1c and 50 to be clinical stage T2 tumours. The RP specimens were examined using fixed and paraffin-embedded sections. The entire specimen was serially blocked at 4-mm intervals with a knife. Tumours were graded according to the Gleason grading system. Extracapsular extension (ECE) was defined as a tumour extending outside of the prostate into the periprostatic soft tissues, so tumour invasion of the prostatic capsule without penetration was not ECE. Seminal vesicle invasion (SVI) was diagnosed when tumour penetrated the muscular coat of the seminal vesicles. Pathologically organ-confined tumours were categorized as pT2 tumours. PNI was diagnosed when there was tumour extension along the perineural sheath. Microvascular invasion (MVI) was diagnosed when the tumour penetrated the endothelial-lined spaces. We did not differentiate lymphatics from blood vessels, as distinguishing them is difficult in the prostate. The tumour border was outlined on the coverslip of the slide using a marking pen. The length of the largest single tumour focus (maximum tumour diameter, MTD) was determined by marking both ends of the lesion with a pen and measuring this distance directly on the glass slide with a ruler. The tumour area on each slide was measured with commercially available software. The area of the largest single tumour focus (maximum tumour area, MTA) was determined. The volume of each cancer was calculated as the sum of the surface areas for that tumour multiplied by the thickness of the prostate slice (total tumour volume, TTV).

The clinical follow-up was 12–99 months; serum PSA levels were measured after RP at least every 3 months. Biochemical failure was defined by an increase in serum PSA levels of >0.2 ng/mL. Cox proportional hazard regression models were used for univariate and multivariate analyses to test the relationships between biochemical failure and pathological factors. For statistical analyses, Gleason score was divided into three groups of ≤6, 7 and ≥8. Continuous tumour dimensional variables (MTD, MTA and TTV) were analysed as dichotomous variables according to 'optimum' thresholds, determined as follows. The value best discriminating between 'good' and 'poor' PSA recurrence-free survival was found by testing all possible thresholds within the central 80% of the distribution of values. All such thresholds were then rounded to clinically convenient values. The *P* values for the clinically relevant thresholds were corrected for multiple testing [8]. Clinicopathological features assessed in univariate analyses include patient age, preoperative PSA level, biopsy Gleason score, pathological T stage, Gleason score, PNI, MVI, surgical margin status, MTD, MTA and TTV. To obtain a multivariate model with maximum precision for the important variables, a stepwise selection procedure was used.

Using the statistically significant variables in the multivariate Cox regression analysis, the relative risk (RR) of biochemical failure were estimated. PSA recurrence-free probabilities for each group were estimated using the Kaplan-Meier method. PSA recurrence was compared between groups using a log-rank test. In all tests, differences were considered statistically significant at $P < 0.05$.

RESULTS

Pathologically, 102 men (62.2%) had disease confined to the prostate (pT2) and 62 (37.8%) had extraprostatic disease (pT3). Among the 62 with extraprostatic disease, ECE was detected in 59 (95%) and 10 (16%) had SVI. The rates of MVI, PNI and positive surgical margins (PSM) in the 164 patients were 44 (26.8%), 117 (71.3%) and 54 (32.9%), respectively; the Gleason score range was 5–9. The mean (range) MTD was 2.0 (0.5–4.9) cm, the MTA 1.8 (0.1–11.0) cm^2 and TTV 3.9 (0.1–33.3) mL, respectively.

In the univariate Cox proportional hazard model, pathologic T stage, Gleason score, PNI, MVI, PSM, MTD, MTA and TTV were significantly associated with biochemical failure (Table 1). In the multivariate Cox proportional hazard model using a stepwise inclusion of these factors, Gleason score, PSM and MTD were independent indices associated with biochemical failure (Table 1).

Using the three statistically significant variables in the multivariate Cox regression analysis (Gleason score, PSM and MTD), we developed a prognostic factor-based model for risk stratification. The RR of PSA recurrence could be calculated as exp [0.85 × MTD + 0.83 × (surgical margin status) + 0.77 or 1.34 × (Gleason score)]. In this equation MTD <2.0 and MTD ≥2.0 equalled 0 and 1, respectively; negative and positive surgical margin status equalled 0 and 1, respectively; Gleason score ≤6 equalled 0, and 7 and ≥8 equalled 1, respectively. The coefficient 0.77 was used for Gleason scores 7 and 1.34 for ≥8. Based on the RR of biochemical failure, patients were divided into three risk groups of low (RR 1.00, 44 men), intermediate (RR 2.17–2.33, 49 men) and high (RR 3.80–20.35, 71 men). The low-risk group consisted of patients with a MTD of <2.0, Gleason score ≤6 and negative surgical margins. The intermediate-risk group consisted of patients with one risk factor (MTD ≥2.0 or Gleason score 7 or PSM). All others were included in the high-risk group. Patients with one risk factor of Gleason score ≥8 were not included in the intermediate group, but were included in the high-risk group as their RR was calculated as 3.80. The 3- and 5-year PSA recurrence-free rates were 100% and 89% in the low-risk group, 77% and 71% in the intermediate group and 42% and 37% in the high-risk group, respectively. The differences among the groups were significant ($P < 0.001$ for low- vs high-risk group, $P = 0.002$ for low- vs intermediate-risk group and $P < 0.001$ for intermediate- vs high-risk group; Fig. 1).

DISCUSSION

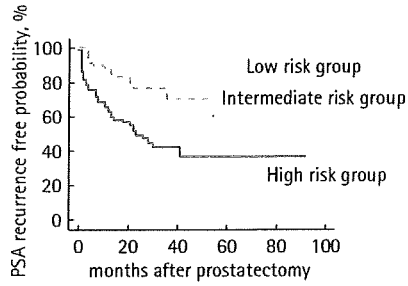
Tumour volume has been thought to be a good candidate for predicting tumour aggressiveness. Previous studies reported on relationships between TTV and PSA failure [9–11]. Although data on tumour volume have been accumulated, it is not still clear whether the tumour volume is an independent predictor of PSA recurrence [12–14]. Some studies showed that tumour volume is an independent predictor of progression [11,15]. Other studies have shown that, although

Variable	Univariate *P*	Multivariate *P*, hazard ratio (95% CI)
Pathological stage	<0.001	–
pT2		
pT3a	0.012	
pT3b	<0.001	
Gleason score	<0.001	0.013
≤6		–, 1.00
7	0.002	0.024, 2.17 (1.11–4.23)
≥8	<0.001	0.005, 3.80 (1.51–9.60)
PNI		
Negative		
Positive	0.022	
MVI		
Negative		
Positive	<0.001	
Surgical margin		
Negative		–, 1.00
Positive	<0.001	0.005, 2.29 (1.28–4.12)
MTD		
≤2.0		–, 1.00
>2.0	<0.001	0.015, 2.33 (1.18–4.61)
MTA		
≤1.3		
>1.3	<0.001	
TTV		
≤2.7		
>2.7	<0.001	

TABLE 1
The univariate and multivariate Cox regression analyses

Gleason score ≤6 was used as the reference for Gleason score, and pT2 for pathological stage.

FIG. 1. PSA recurrence-free rates according to the three risk groups (low-risk, MTD <2.0, Gleason score ≤6 and negative surgical margin; intermediate risk, one risk factor, i.e. MTD ≥2.0 or Gleason score 7, or PSM; all others in the high-risk group). Patients with one risk factor of Gleason score ≥8 were not included in the intermediate group, but in the high-risk group.



tumour volume was a significant univariate predictor of progression, it did not provide additional prognostic information if other pathological features were known [9,10,16]. In the present study, tumour dimensional variables, including MTD, MTA and TTV, were significant predictors of biochemical recurrence in univariate analyses. It is not surprising that they directly reflect PSA recurrence because it was shown that MTD, MTA and TTV were closely associated with other pathological features [17]. As all tumour dimensional variables are correlated with biochemical recurrence, we consider that their estimation is desirable in patients with prostate cancer. Among these variables, MTD was an independent predictor of biochemical recurrence in clinically localized prostate cancer even in multivariate analysis. Possible explanations for these results include differences in calculating tumour foci. TTV is a sum of tumour volumes when the tumours are multifocal and seems to reflect total tumour activity. On the other hand, MTD seems to reflect a character of the largest focus of the tumours. In fact MTD seems to be along the prostate capsule in many cases and it seems likely that the probability of a tumour having ECE is related to the extent of its presence at the capsular surface. Perhaps the largest focus of tumours, which have MTD, is more closely associated with local extent of disease than TTV. Some investigators reported that the index tumour volume was equally predictive of PSA recurrence as TTV [15,18]. Similar to those studies, our results suggest that, even though most large tumours are multifocal, tumour aggressiveness might depend most on the largest focus. Every sample we used in the present study was a whole-mount step section, but different centres might process their RP specimens differently. In some whole-mount sections there seems to be difficulty in calculating tumour size variables exactly, especially in cases with index cancer lying between bilateral lobes. We believe the threshold value assessed in the present study will help to assess those cases to some extent.

Biochemical failure is relatively common in patients treated with RP [1,19]; of those with biochemical recurrence, 34% will develop metastatic disease [1]. Therefore, identifying patients with early treatment failure is important for patient management. Our multivariate analysis showed that surgical margin status and Gleason score independently predicted biochemical recurrence after RP, in addition to the MTD status. Using independent predictors in multivariate analysis we established a prognostic factor- based risk stratification for clinically localized prostate cancer. Patients were stratified into three groups according to statistical modelling based on the RR. From this result we consider that patients presenting with those risk factors require a close follow-up for the early detection of biochemical failure, or might be candidates for a clinical trial of adjuvant or innovative treatment. Our results provide useful information to predict biochemical failure in patients treated with RP.

In conclusion, the present results imply that MTD is possibly a most important prognostic factor for predicting biochemical recurrence after RP. As calculating the MTD from whole-mount sections is rapid, simple, inexpensive and objective, it can be used instead of calculating the TTV. The prognostic factor-based risk stratification might help clinicians to predict biochemical failure.

CONFLICT OF INTEREST

None declared.

REFERENCES

1 **Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, Walsh PC.**

Natural history of progression after PSA elevation following radical prostatectomy. *JAMA* 1999; **281**: 1591–7
2 **Humphrey PA, Vollmer RT.** Percentage carcinoma as a measure of prostatic tumor size in radical prostatectomy tissues. *Mod Pathol* 1997; **10**: 326–33
3 **McNeal JE, Bostwick DG, Kindrachuk RA, Redwine EA, Freiha FS, Stamey TA.** Patterns of progression in prostate cancer. *Lancet* 1986; **1**: 60–3
4 **Eichelberger LE, Koch MO, Eble JN, Ulbright TM, Juliar BE, Cheng L.** Maximum tumor diameter is an independent predictor of prostate-specific antigen recurrence in prostate cancer. *Mod Pathol* 2005; **18**: 886–90
5 **Renshaw AA, Chang H, D'Amico AV.** Estimation of tumor volume in radical prostatectomy specimens in routine clinical practice. *Am J Clin Pathol* 1997; **107**: 704–8
6 **Renshaw AA, Richie JP, Loughlin KR, Jiroutek M, Chung A, D'Amico AV.** The greatest dimension of prostate carcinoma is a simple, inexpensive predictor of prostate specific antigen failure in radical prostatectomy specimens. *Cancer* 1998; **83**: 748–52
7 **Renshaw AA, Richie JP, Loughlin KR, Jiroutek M, Chung A, D'Amico AV.** Maximum diameter of prostatic carcinoma is a simple, inexpensive, and independent predictor of prostate-specific antigen failure in radical prostatectomy specimens. Validation in a cohort of 434 patients. *Am J Clin Pathol* 1999; **111**: 641–4
8 **Atzpodien J, Royston P, Wandert T, Reitz M.** Metastatic renal carcinoma comprehensive prognostic system. *Br J Cancer* 2003; **88**: 348–53
9 **Epstein JI, Carmichael M, Partin AW, Walsh PC.** Is tumor volume an independent predictor of progression following radical prostatectomy? A multivariate analysis of 185 clinical stage B adenocarcinomas of the prostate with 5 years of followup. *J Urol* 1993; **149**: 1478–81
10 **Ohori M, Kattan M, Scardino PT, Wheeler TM.** Radical prostatectomy for carcinoma of the prostate. *Mod Pathol* 2004; **17**: 349–59
11 **Carvalhal GF, Humphrey PA, Thorson P, Yan Y, Ramos CG, Catalona WJ.** Visual estimate of the percentage of carcinoma is an independent predictor of prostate carcinoma recurrence after radical prostatectomy. *Cancer* 2000; **89**: 1308–14
12 **Epstein JI, Partin AW, Sauvageot J, Walsh PC.** Prediction of progression following radical prostatectomy. A multivariate analysis of 721 men with long-term follow-up. *Am J Surg Pathol* 1996; **20**: 286–92
13 **Humphrey PA, Frazier HA, Vollmer RT, Paulson DF.** Stratification of pathologic features in radical prostatectomy specimens that are predictive of elevated initial postoperative serum prostate-specific antigen levels. *Cancer* 1993; **71**: 1821–7
14 **Humphrey PA, Vollmer RT.** Intraglandular tumor extent and prognosis in prostatic carcinoma: application of a grid method to prostatectomy specimens. *Hum Pathol* 1990; **21**: 799–804
15 **Stamey TA, McNeal JE, Yemoto CM, Sigal BM, Johnstone IM.** Biological determinants of cancer progression in men with prostate cancer. *JAMA* 1999; **281**: 1395–400
16 **Kikuchi E, Scardino PT, Wheeler TM, Slawin KM, Ohori M.** Is tumor Volume an independent prognostic factor in clinically localized prostate cancer? *J Urol* 2004; **172**: 508–11
17 **Mizuno R, Nakashima J, Mukai M *et al.*** Maximum tumor diameter is a simple and valuable index associated with the local extent of disease in clinically localized prostate cancer. *Int J Urol* 2006; **13**: 951–5
18 **Wise AM, Stamey TA, McNeal JE, Clayton JL.** Morphologic and clinical significance of multifocal prostate cancers in radical prostatectomy specimens. *Urology* 2002; **60**: 264–9
19 **Han M, Partin AW, Zahurak M, Piantadosi S, Epstein JI, Walsh PC.** Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. *J Urol* 2003; **169**: 517–23

Correspondence: Jun Nakashima, Department of Urology, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, Japan.
e-mail: njun@tokyo-med.ac.jp

Abbreviations: **RP**, radical prostatectomy; **PSM**, positive surgical margin; **MTD**, maximum tumour diameter; **MTA**, maximum tumour area; **TTV**, total tumour volume; **RR**, relative risk; **SVI**, seminal vesicle invasion; **PNI**, perineural invasion; **ECE**, extracapsular extension; **MVI**, microvascular invasion.

Original Paper

Pathobiology

Pathobiology 2009;76:293–302
DOI: 10.1159/000245895

Received: March 16, 2009
Accepted after revision: June 17, 2009

Phosphorylation Status of Fas-Associated Death Domain-Containing Protein Regulates Telomerase Activity and Strongly Correlates with Prostate Cancer Outcomes

Yoshiaki Matsumura[a] Keiji Shimada[b] Nobumichi Tanaka[a] Kiyohide Fujimoto[a]
Yoshihiko Hirao[a] Noboru Konishi[b]

Departments of [a]Urology and [b]Pathology, Nara Medical University, Nara, Japan

Key Words
FADD phosphorylation · hTERT · Prostate cancer

Abstract
Objectives: We investigated whether the phosphorylated Fas-associated death domain protein (FADD) at serine 194 regulated human telomerase reverse transcriptase (hTERT) expression, telomerase activity and cancer progression using prostate cancer cell lines and radical prostatectomy samples taken from patients receiving neoadjuvant hormonal therapy (NHT). ***Methods:*** We analyzed hTERT expression, telomerase activity and invasion capacity in prostate cancer cell lines overexpressing the wild-type or mutant form of FADD (S194D or A). FADD, phosphorylated FADD (p-FADD) and hTERT expression in viable prostate cancer cells following NHT were immunohistochemically examined using 50 prostatectomy samples. ***Results:*** Dephosphorylated FADD (S194A) overexpression enhanced hTERT expression and telomerase activity, resulting in increased cell proliferation and invasion capacity. In Kaplan-Meier survival analysis, the patients with prostate cancer expressing low levels of p-FADD and high levels of hTERT had significantly higher rates of biochemical recurrence than those with high p-FADD and low hTERT expression ($p < 0.001$). ***Conclusions:*** The phosphorylation status of FADD at serine 194 could strongly affect survival and invasion of prostate cancer cells via modulation of hTERT expression and telomerase activity. p-FADD and hTERT expression may have potential as new biomarkers predicting the biochemical recurrence after NHT.

Introduction

Prostate cancer is one of the most commonly diagnosed cancers, and one of the leading causes of cancer-related deaths around the world, including Japan. The rationale for combined modality treatment, such as radical prostatectomy and external beam radiation therapy with neoadjuvant hormonal therapy (NHT), has recently attracted attention, although not yet established in the routine clinical practice. For patients undergoing radical prostatectomy, NHT has reportedly achieved good results [1]. However, NHT does not yield any improvement in the overall and disease-free survival [2], and it remains

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015–2008/09/0766–0293$26.00/0

Accessible online at:
www.karger.com/pat

Dr. Yoshiaki Matsumura
Department of Urology, Nara Medical University
840 Shijo-cho, Kashihara, Nara 634-8522 (Japan)
Tel. +81 744 22 3051, ext. 2238, Fax +81 744 22 9282
E-Mail ymatsumu@tcc.pref.tochigi.jp

controversial whether NHT before radical prostatectomy is useful for cancer control. Moreover, we have no biological markers to assess the malignant potential of viable cancer cells (so-called hormone-resistant cells) following NHT to predict cancer progression. Unfortunately, morphological assessment of the therapeutic effects using radical prostatectomy specimens does not significantly correlate with the biochemical recurrence rate.

The Fas-associated death domain-containing protein (FADD) was originally identified as an adapter molecule for Fas-mediated apoptosis [3, 4], and it is now well known to play an important role in the formation of the death-inducing signaling complex following Fas stimulation [5]. Recent reports have shown that FADD is exclusively phosphorylated at the C-terminal serine 194, specifically at the G2/M cell cycle arrest, suggesting a close association with cell cycle regulation [6]. FADD is phosphorylated at serine 194, which is implicated in cell cycle progression but not associated with the sensitivity to Fas-mediated apoptosis. Analysis of FADD mutant null mice revealed that FADD also plays a role in embryonic development, cell cycle control and proliferation of lymphoid cells [7, 8]. Moreover, we have demonstrated that the phosphorylated FADD (p-FADD) is highly expressed in prostate cancer cells of lower Gleason score using prostatectomy samples without NHT [9]. Taken together, FADD phosphorylation could lead to suppression of prostate cancer progression. However, the biological role of the dephosphorylated FADD in prostate cancer cells has not been identified yet. Moreover, it remains unclear whether the phosphorylation status of FADD is modified in response to NHT or not. We focused on human telomerase reverse transcriptase (hTERT), which is involved in cancer growth [10], as the downstream molecule of the dephosphorylated FADD.

In this study, we investigated whether the phosphorylation status and hTERT expression in cancer cells could be useful biomarkers to estimate the malignant potential and to predict the prognosis of human prostate cancers, and moreover, whether these molecules might be the best therapeutic targets.

Materials and Methods

Cell Culture, Plasmids and Chemicals

Human prostate cancer cell lines (PC-3) were purchased from American Type Culture Collection (Manassas, Va., USA) and cultured in RPMI supplemented with 10% fetal bovine serum. FLAG-tagged human FADD and mutant FADD: S194→A (dephosphorylation-mimicking form) and S194→D (phosphorylation-mimicking form) were prepared as described previously [11]. Anti-wild-type FADD (WT-FADD) was purchased from BD Pharmingen (Tokyo, Japan); anti-p-FADD at S194 from Cell Signaling (Beverly, Mass., USA) and anti-hTERT from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted with Trizol reagent and subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the one-step RT-PCR kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The PCR condition was at 94°C for 1 min, at 59°C (hTERT)/60°C (GAPDH) for 30 s and at 72°C for 1 min, for 35 cycles. Human specific primers, which we designed using the Primer Quest program, were purchased from IDT (Coralville, Iowa, USA). hTERT-F, 5′-CTACTCCTCAGGCGACAAGG-3′; hTERT-R, 5′-TGGAACCCAGAAAGATGGTC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F, 5′-ACCACAGTCCATGCCATCAC-3′, and GAPDH-R, 5′-TCCACCACCCTGTTGCTGTA-3′ were used. The PCR products were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining.

Telomeric Repeat Amplification Protocol Assay

The cells were homogenized in lysis buffer and 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, and incubated on ice for 30 min. The cell lysate was clarified by centrifugation at 14,000 *g* for 20 min at 4°C, and the supernatants were freshly frozen at –80°C. Telomerase activity was determined using the TRAP-eze telomerase detection kit S7700 kit (Chemicon International, Temecula, Calif., USA). The gel was stained with silver using Ezstain Silver (Atto, Tokyo, Japan).

Chick Chorioallantoic Membrane Assay

In vivo cancer cell invasion and intravasation assays were carried out using 11-day-old chick embryos wherein 10^6 cancer cells transfected with the green fluorescent protein (GFP)-encoding vector pEGFP (Clontech, Palo Alto, Calif., USA) with FLAG-tagged human FADD and mutant FADD (S194A FADD and S194D FADD). After 24-hour culture, the cells were seeded on the chorioallantoic membrane (CAM) for 3 days [12]. After collection of the CAM samples, the tissues were fixed and stained with anti-GFP. Thereafter, the number of invading cancer cells positive for GFP was counted in three or more randomly selected fields. At least three CAMs were used in each experiment.

Cell Viability Assay

The cells were cultured in a medium containing fetal bovine serum for 4 days. After incubation, MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H tetrazolium, inner salt] reagent (Promega, Tokyo, Japan) was added, and optical absorbance at 490 nm was measured every day using a microplate reader [11].

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

We detected DNA cleavage, a characteristic of apoptosis, using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, as directed by the manufacturer (GenScript, Piscataway, N.J., USA). Images of nuclear fluorescence typical of apoptotic cells were taken by fluorescence microscopy as previously described, examining at least 600 cells

Table 1. Patient characteristics at diagnosis

Characteristics	Biochemical recurrence		p value
	yes (n = 18)	no (n = 32)	
Mean age, years	69.6	69.4	0.71
PSA	41.1	16.5	
≤10 ng/ml	2 (11%)	14 (44%)	
10.1–20 ng/ml	3 (17%)	12 (38%)	
≥20 ng/ml	13 (72%)	6 (19%)	0.001
Gleason score			
≤6	2 (11%)	15 (47%)	
7	10 (56%)	5 (16%)	
8–10	5 (28%)	10 (31%)	
Unknown	1 (6%)	2 (6%)	0.015
Clinical stage			
T1c	0	12 (38%)	
T2a-b	12 (67%)	15 (47%)	
T3–4	6 (33%)	5 (16%)	0.010

from three different fields in each experiment; cell death was expressed as percentage of the TUNEL-positive cells [13].

Patients and Treatment

We used tissue specimens from 50 patients considered as non-effective cases of NHT who underwent radical prostatectomy following NHT between 1997 and 2004 at our hospital. 'Non-effective' case means that almost all cancer cells were viable or less than one half of cancer cells were non-viable. The patients' age ranged from 53 to 75 (median: 70.5) years. The median prostate-specific antigen (PSA) level at biopsy was 13.95 ng/ml (range: 6.0–36.3 ng/ml; Tandem-R assay). NHT consisted of maximum androgen blockade with a luteinizing hormone-releasing hormone agonist either alone (8 patients/16%) or combined with an anti-androgen agent (42 patients/84%) The median NHT duration was 6.6 (range: 4–12) months. All patients underwent serum PSA testing at least every 3 months after surgery. A PSA level ≥0.2 ng/ml after surgery was defined as biochemical recurrence. The median follow-up period after radical prostatectomy was 45.7 (range: 17–102) months. The Human Ethics Review Committee of Nara Medical University approved the study protocol. The PSA levels at diagnosis, the Gleason score at biopsy and the clinical T stage are shown in table 1.

Immunohistochemistry

The sections were incubated for 16 h at 4°C, and the reactions were visualized using a Histofine SAB-PO kit and diaminobenzidine as chromogen (Nichirei, Tokyo, Japan), with hematoxylin counterstaining.

Statistical Analysis

Fisher's exact probability tests were used for the assessment in the proliferation assay and the clinical parameters of biochemical recurrence. Wilcoxon signed-rank tests were used to evaluate the invading cancer cell numbers of mutant FADD and the relationship between the percentages of p-FADD-positive cells and the intensity of hTERT expression. Kaplan-Meier survival curves and log-rank tests were used for survival analysis of biochemical recurrence. The results were considered significant if $p < 0.05$.

Results

Phosphorylation Status of FADD at S194 Correlates with hTERT Expression and Telomerase Activity

To clarify the effect of the dephosphorylated form of FADD on telomerase activity, we constructed PC-3 cells stably overexpressing FLAG-tagged plasmids encoding the wild-type and mutant form of FADD, in which serine 194 was replaced by alanine (S194A, mimicking dephosphorylated FADD) or glutamate (S194D, mimicking p-FADD). Among several clones resistant to hygromycin B, we selected a single clone fully expressing the wild-type or mutant forms of FADD, as determined by immunoblotting using anti-FLAG antibody (fig. 1A). The level of expression of hTERT mRNA and protein was minimal in the control clone (expressing hygromycin-B-resistant gene only), but it was greatly increased by overexpression of the wild-type or S194A FADD, resulting in enhancement of the telomerase activity in PC3 cells (fig. 1B, C). In the clones overexpressing S194D FADD, both hTERT expression and telomerase activity were not significantly changed, indicating that the dephosphorylated form of FADD might play an essential role in the telomerase activity through inducing hTERT in prostate cancer cells.

Phosphorylation Status of FADD at S194 Regulates Prostate Cancer Proliferation, Invasion and Sensitivity to Anti-Cancer Agents

Next, we examined the effects of the phosphorylation status of FADD at serine 194 on cell proliferation by MTT assay. As shown in figure 2A, the cell number increased to the same extent within the 48-hour incubation in the controls and clones expressing the wild-type and mutant form of FADD. However, thereafter, the proliferation rate of cells was much more increased in the clones expressing the wild-type or S194A FADD than in those with S194D FADD expression or the control. Finally, the effects of FADD phosphorylation on cancer invasion were analyzed using the in vivo CAM assay. The cells were transfected with vector encoding GFP for 12 h, and then implanted on CAM. After 5 days, the membranes were extracted and the invasion capacity was assessed by examining the number of GFP-pos-

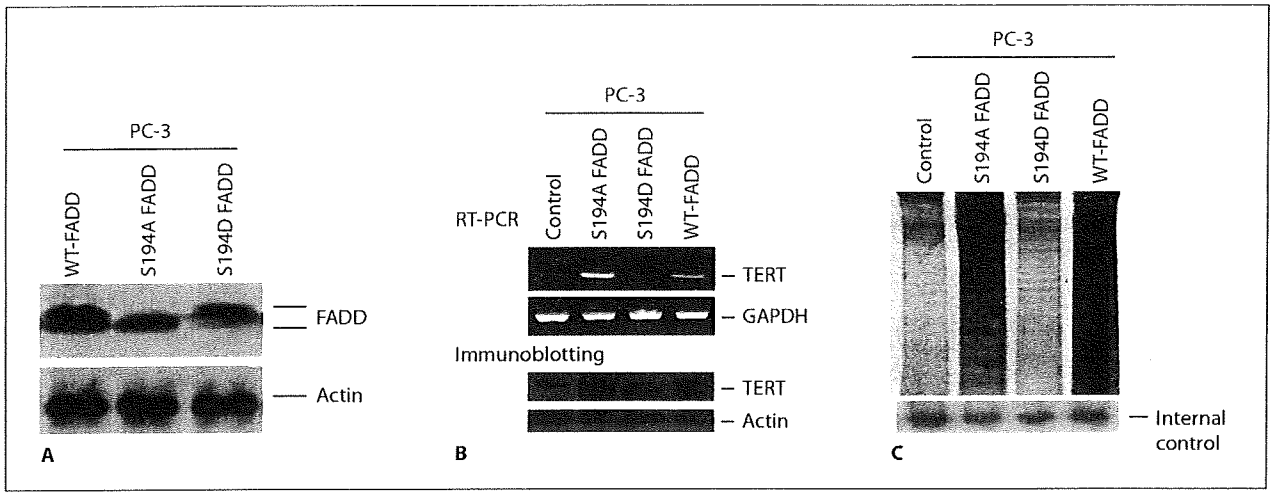


Fig. 1. The phosphorylation status of FADD regulated hTERT expression and telomerase activity. The sequence of the C-terminal domain of FADD harbors key phosphorylation sites at serine 194. PC-3 cells were transfected with the hygromycin B resistance gene with a FLAG-tagged vector encoding S194A and S194D mutant FADD and WT-FADD. **A** Whole lysates overexpressing A194, D194 and WT were examined by Western blotting using anti-FADD and anti-actin antibodies. **B** RNA was extracted, and RT-PCR assays were done to detect hTERT mRNAs. Whole lysates were examined by Western blotting using anti-TERT and anti-actin antibodies. **C** Cell pellets were collected and subjected to TRAP assay.

itive invading cells as identified by green fluorescence (fig. 2B). The invading cell numbers were significantly increased by the wild-type or S194A FADD overexpression but not by S194D FADD (fig. 2C). When we obtained graft samples on day 3 after implantation, the numbers of invading cells were not significantly different among these clones, being consistent with the results of the in vitro MTT assay (data not shown). Taken together, the dephosphorylated form of FADD could enhance cell growth and invasion capacity in human prostate cancer cells. In addition, we examined the sensitivity of anti-cancer agents in the clone overexpressing empty vector, WT-FADD, S194A and S194D using TUNEL staining. The sensitivities of PC3 cells to cisplatin and etoposide were greatly increased by overexpression of the wild-type and S194D FADD. Moreover, the sensitivities to cisplatin and etoposide in the clones expressing S194D FADD were higher than in the wild type. In contrast, in the clones overexpressing S194A FADD, the sensitivities to cisplatin and etoposide were not significantly changed, indicating that the phosphorylated form of FADD might induce the susceptibility to anti-cancer agents (fig. 2D). Nonetheless, the dephosphorylated FADD did not reduce the susceptibility to anti-cancer agents because PC-3 is originally less sensitive to the anti-cancer agents. To clarify the induction of apoptosis by FADD transfection, we examined the apoptotic status by TUNEL staining. The transfection of FADD induced apoptosis, but the rates of apoptosis were only minimal. Besides, induction of apoptosis was not significantly different between the clones expressing WT-FADD, S194A FADD and S194D FADD (data not shown).

Expression of Phosphorylated FADD/hTERT in Prostate Cancer Cells and Biochemical Recurrence following NHT

FADD, p-FADD and hTERT expressions were immunohistochemically analyzed using radical prostatectomy samples following NHT. As shown in figure 3A, FADD was equally expressed in the cases with and without biochemical recurrence. Nevertheless, p-FADD expression at serine 194 was increased in cancer cells without recurrence. Phosphorylated FADD expression was considered positive when >15% (median level in all specimens) of the cancer cells showed positive immunostaining. As demonstrated in figure 3B, Kaplan-Meier analysis showed that the biochemical recurrence-free survival rate of the p-FADD-positive cases was significantly higher than that of the -negative cases ($p < 0.001$).

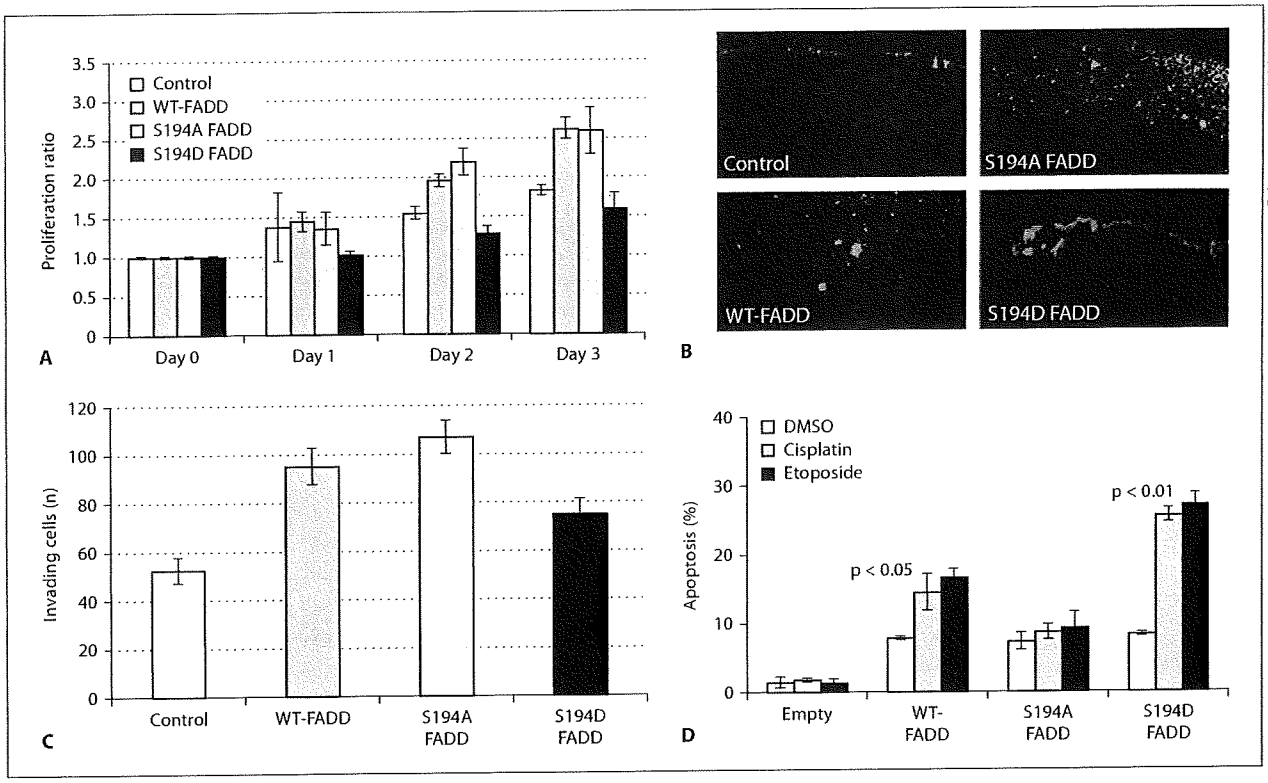
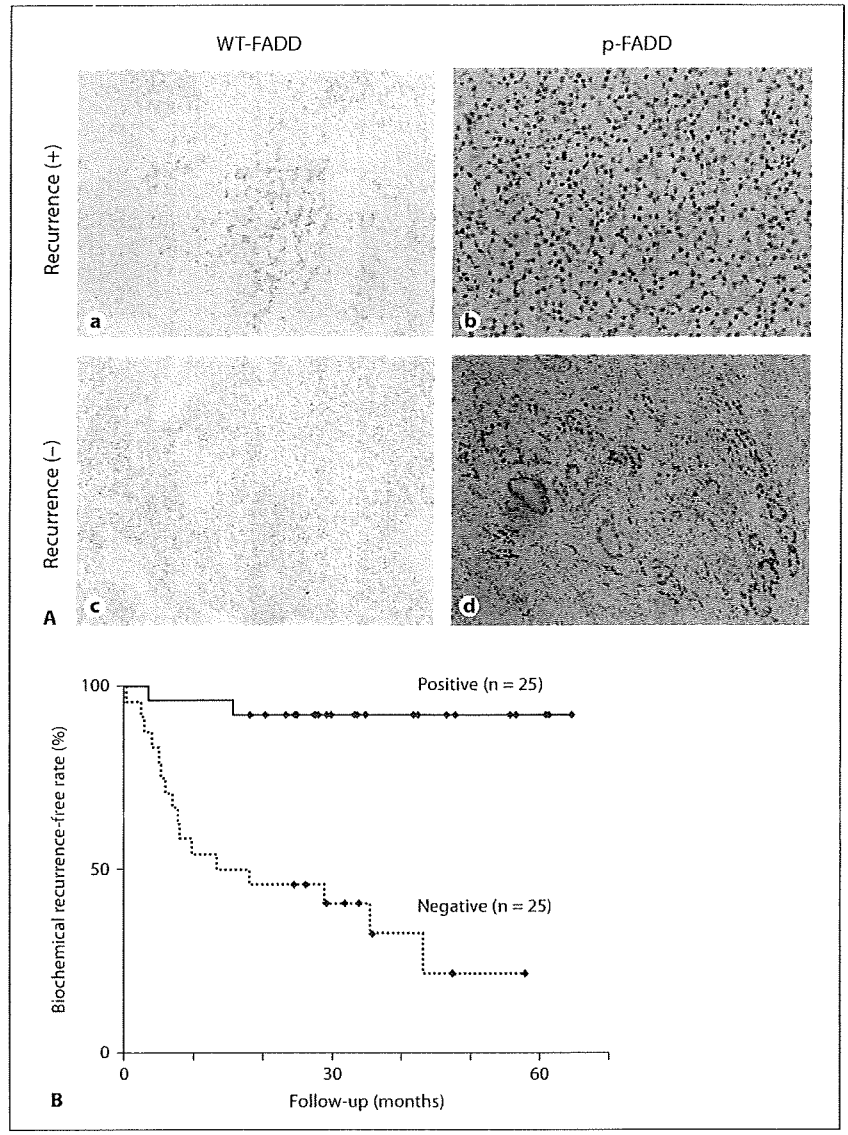


Fig. 2. Phosphorylation status of FADD at serine 194 regulated the cell proliferation and invasion in prostate cancer cells. **A** PC-3 cells were co-transfected with a β-gal-encoding vector with and without FLAG-tagged WT-FADD and mutant FADD (S194A and S194D). After a 1-day incubation, cell survival after 2, 3 and 4 days in the medium was analyzed by MTT assay. The experiments were performed at least in triplicate. **B, C** PC-3 cells were transfected with GFP-encoding plasmids in the presence of control, WT-FADD, S194A mutant FADD and S194D mutant FADD. After 24 h in culture, cells were plated on CAM, and grafts were collected after 3 days. The tissues were fixed and stained with anti-GFP, and, thereafter, the number of invading GFP-positive cancer cells was quantified in three or more randomly selected fields. **D** p-FADD at serine 194 induced susceptibility to anti-cancer agents. After a 48-hour incubation with 10 μM cisplatin and etoposide, the apoptotic rates induced by these agents were analyzed by TUNEL stain.

In contrast to the p-FADD, hTERT was not or little expressed in the cancer cells without recurrence, whereas it was highly expressed in those with biochemical recurrence (fig. 4A). In the present study, the intensity of immunostaining of hTERT was divided into three levels (mild, moderate and strong), and it closely correlated with protein expression levels assessed by immunoblotting (fig. 4B). Furthermore, the biochemical recurrence-free survival rate of the patients with low levels of hTERT was significantly greater than in those with high-level expression ($p < 0.001$; fig. 4C). There was a significant inverse correlation between the percentages of the p-FADD-positive cells and the staining intensity of hTERT as follows: the positive percentage of the p-FADD was 28.9 ± 12.1, 15.6 ± 10.8 and 10.5 ± 6.1% in the mild, moderate and strong intensities of hTERT, respectively (fig. 5).

Discussion

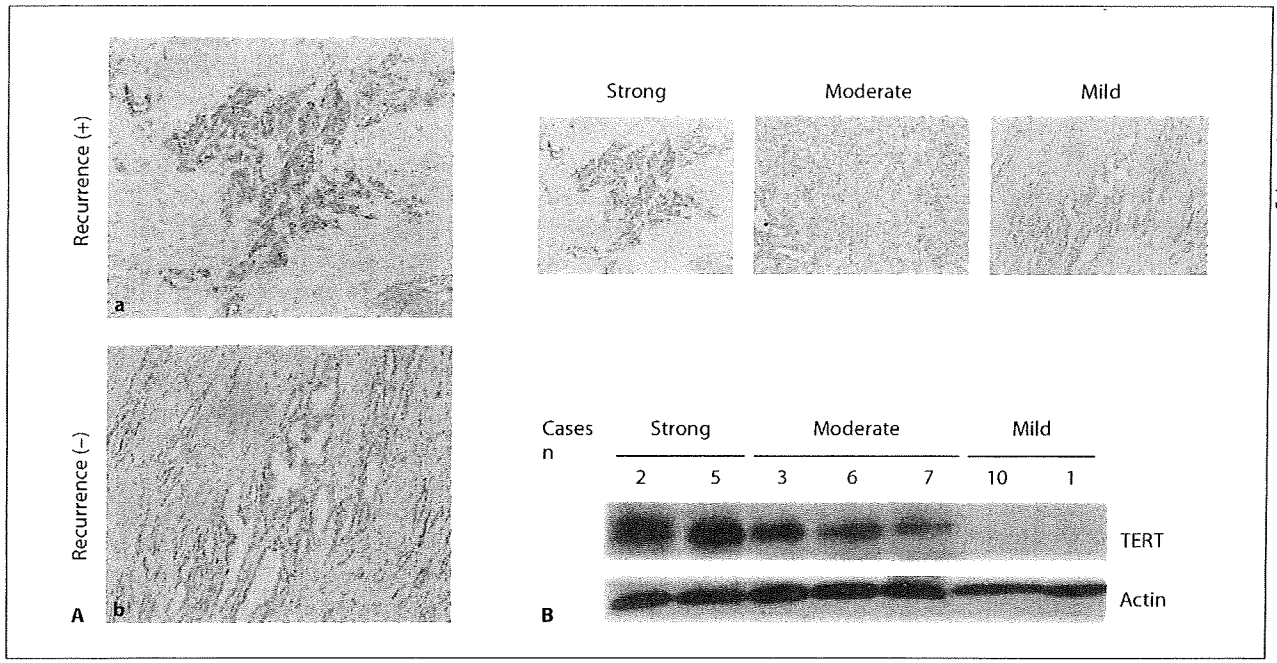
We have already demonstrated that transition from dephosphorylation to phosphorylation of FADD at serine 194 could lead to cell cycle arrest and growth suppression in human prostate and breast cancer cells [9]. However, the biological roles of dephosphorylated FADD in prostate cancer cells have not been investigated yet. In our present in vitro experiments using the wild-type or



Color version available online

Fig. 3. A Phosphorylation status of FADD at serine 194 in prostate cancer cells. The specimens were obtained from patients undergoing radical prostatectomy following NHT with (**a**, **b**) and without biochemical recurrence (**c**, **d**), and were stained with anti-FADD (**a**, **c**) and anti- p-FADD (**b**, **d**) antibodies. **a**, **c** ×100. **b**, **d** ×200. **B** Kaplan-Meier biochemical recurrence-free survival curve according to p-FADD expression (log-rank test: $p < 0.001$). p-FADD expression was considered positive when >15% (median level in all specimens) of the cancer cells showed positive immunostaining.

phosphorylation-/dephosphorylation-mimicking mutant forms of FADD and human prostate cancer cell lines, we clearly demonstrated for the first time that the dephosphorylated FADD significantly enhanced cancer cell growth through inducing hTERT expression. Telomerase is a ribonucleoprotein enzyme with specialized reverse transcriptase activity that catalyzes the synthesis and extension of telomeric DNA [14, 15]. The telomerase complex is composed of the catalytic subunit reverse transcriptase protein hTERT [16], the telomerase RNA template subunit, hTR [17] and other associated proteins [18]. Telomerase activity is typically absent in most normal human cells, but it is expressed in nearly all human cancer cells. Besides, a strong correlation is observed between hTERT mRNA expression and telomerase activity in various epithelial cancers indicating that hTERT may be mostly transcriptionally regulated [19]. The activity of telomerase, hTERT induction and cancer cell proliferation were accelerated by overexpression of the wild-type and dephosphorylated mutant FADD. Moreover, it was more prominent in cases showing overexpression of the dephosphorylated mutant FADD (S194A) but not the



Color version available online

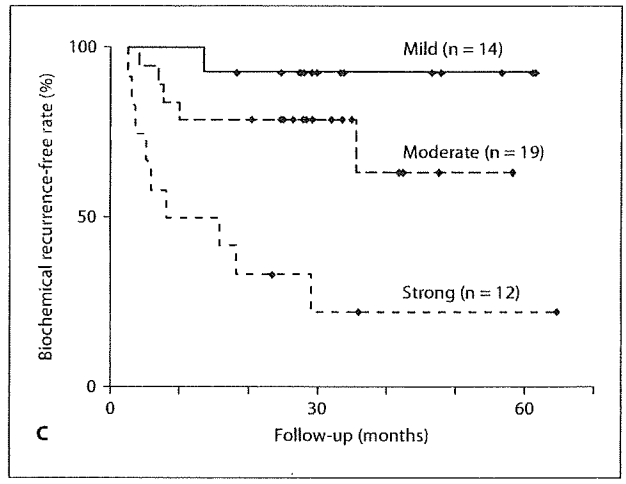


Fig. 4. hTERT expression in prostate cancer cells. **A** The specimens were obtained from patients undergoing radical prostatectomy following NHT with (**a**) and without biochemical recurrence (**b**). They were stained with anti-TERT antibodies. ×100. **B** Immunohistochemical staining intensity of hTERT was divided into three levels (mild, moderate and strong). Immunoblotting analysis of TERT protein expression in the specimens from the patients undergoing radical prostatectomy and NHT. **C** Kaplan-Meier biochemical recurrence-free survival curve according to the hTERT expression (log-rank test: $p < 0.001$).

phosphorylated mutant FADD (S194D). Similar results were also observed regarding the invasion capacity as follows: the invading cell number of the prostate cancer cell line was significantly increased compared with the wild-type and dephosphorylated FADD, but not by the p-FADD. These FADD-mediated effects on cell proliferation and invasion were strongly suppressed by silencing of hTERT by siRNA transfection (data not shown). hTERT induction and its dependent increase in telomerase activity are considered to be mainly involved in the mechanisms. To verify this hypothesis, we examined several molecules associated with invasion or migration, including matrix metalloproteinases 2 and 9, and urokinase-type plasminogen activator, but the protein/mRNA levels or gelatinolytic activity was not significantly changed by FADD overexpression. In addition, the fold increase in cell proliferation by FADD transfection was almost similar to that of the invading cell number (fig. 2A, C). Therefore, FADD status-mediated enhancement of cancer invasion is closely dependent on the in-

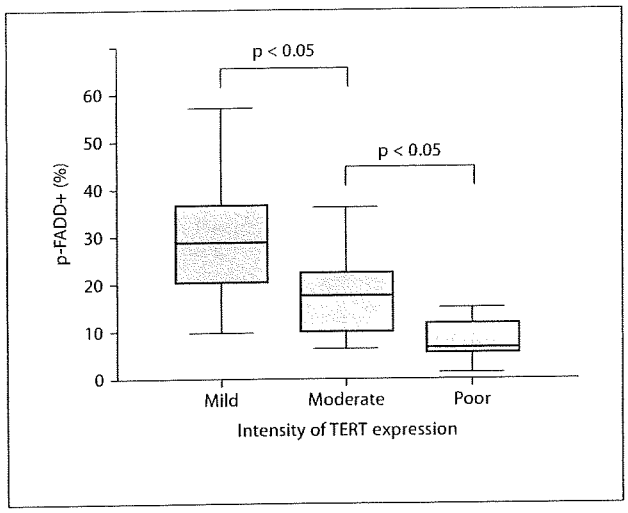


Fig. 5. Box plots showing the correlation between the positivity for the p-FADD and the intensity of hTERT.

crease in cell growth. FADD is a well-known key molecule that mediates apoptosis induction by Fas, tumor necrosis factor (TNF) or TNF-related apoptosis-inducing ligands, and the cells lacking FADD show resistance to receptor-mediated cell death [20]. In contrast, Chen et al. [21] have recently provided evidence for a strong correlation between overexpression of FADD mRNA and protein in human lung adenocarcinomas and a poor clinical outcome. Furthermore, Amit et al. [22] demonstrated that FADD could induce NF-κB, which plays a central role in tumor development, progression and therapy. Taken together, FADD and the dependent signals have suppressive and promotive effects on human cancer [23]. In contrast to our present data, Chen et al. [21] indicated that the phosphorylated form of FADD induces NF-κB, perturbs the cell cycle and is associated with progression of the biological behavior of lung adenocarcinomas. A recent study on acute myeloid leukemia has shown that the absence or low expression of FADD protein is a poor prognostic factor [24]. These contradictory results might be due to cell-type-specific aberrations in dichotomous FADD-mediated pathways that regulate both cell growth and apoptosis. Studies using immunohistochemistry and subcellular fractionation analysis revealed that FADD is a predominantly nuclear protein in many cell lines [25, 26]. Our present results showed that the phosphorylated form of FADD was localized in the nucleus, whereas FADD was localized in both the cytoplasm and nucleus (fig. 3A). Consequently, we speculate there are key proteins localized in the cytoplasmic fraction that can interact with the dephosphorylated FADD and translocate into the nucleus followed by acceleration of hTERT transcription. As we still have no conclusive evidence, this issue should be further evaluated. We found no mutation at serine194 in prostate cancer specimens in the present study (data not shown). Therefore, transition from dephosphorylation to phosphorylation might be the best target for a useful cancer therapy. Taxanes are reportedly one of the candidates that can successfully phosphorylate FADD at serine194 [9, 27]. Since taxanes including docetaxel are useful anti-cancer drugs for prostate cancer and are currently used worldwide [28, 29], FADD phosphorylation can be a biomarker to evaluate the therapeutic outcome.

A more important finding in the present study is that the p-FADD at serine 194 and hTERT expression are available biomarkers to predict the biochemical recurrence following neoadjuvant androgen withdrawal therapy. Increased positive percentage for the p-FADD and low expression of hTERT are significantly associated with a good prognosis following NHT. Furthermore, there was a strong correlation between the percentages of positive cells for p-FADD and the intensity of hTERT, and this is consistent with our analyses in vitro and in vivo. The prostate cancer cells used in the immunohistochemical analysis were all morphologically determined as 'viable' cells, and therefore, these biomarkers can estimate the clinical effects of NHT independently from the morphological or pathological assessment.

To date, there are no useful tools to analyze the therapeutic effects using radical prostatectomy samples following NHT other than the pathological methods. However, it is well known that the morphological analysis tends to lack reproducibility and reliability, because due to marked degeneration of the remaining cancer cells, we cannot accurately assess whether the cells have low or high malignant potential or no malignancy by pathological examinations only. In fact, we have encountered cases with good outcome although the pathological assessment showed poor response to NHT. From the present data, the positivity for the p-FADD and hTERT could be a novel marker to determine whether the therapeutic response is good or not. In our previous report, WT-FADD expression in both normal prostate epithelial cells and cancer cells was not significantly different [9]. The present study showed that the expression of WT-FADD in a patient with biochemical recurrence was equal to that in recurrence-free cases. Thus, we suggested that

dephosphorylated FADD could enhance hTERT expression of prostate cancer following NHT. Furthermore, induction of dephosphorylated FADD to p-FADD, i.e. reduction of the dephosphorylated FADD by NHT, might improve the therapeutic benefit. On the other hand, no induction of p-FADD by NHT was associated with a poor prognosis. In addition, p-FADD enhanced the sensitivity to anti-cancer agents, but dephosphorylated FADD did not change it (fig. 2D). We found that FADD phosphorylation at Ser194 was associated with paclitaxel-induced upregulation of MEK kinase 1 expression and enhancement of the downstream c-jun NH2-terminal kinase (JNK) pathway by which prostate cancer cells were highly sensitized to apoptosis by etoposide or cisplatin. We speculated that the enhancement of TERT modulating the dephosphorylated FADD could implicate inhibition of JNK activation or inhibition of the JNK induction of apoptosis.

The initial purpose of this study was to determine a useful predictor of biochemical recurrence in patients who underwent radical prostatectomy with NHT. A decade ago, lots of patients received NHT prior to radical prostatectomy. Indeed, 48.5% of patients had NHT at our institute between 1997 and 2004. Nowadays, most patients who undergo radical prostatectomy are hormone naïve. Therefore, an additional study similar to this one is currently being conducted on hormone-naïve patients who underwent radical prostatectomy.

In summary, the transition from the p-FADD to the dephosphorylated form of FADD induced the activation of telomerase through hTERT transcriptional activity. Moreover, p-FADD and hTERT expression have potential as new biomarkers to predict the rate of biochemical recurrence in patients undergoing radical prostatectomy following NHT.

References

1 Witjes WP, Schulman CC, Debruyne FM: Preliminary results of a prospective randomized study comparing radical prostatectomy versus radical prostatectomy associated with neoadjuvant hormonal combination therapy in T2–3 N0 M0 prostatic carcinoma. The European Study Group on Neoadjuvant Treatment of Prostate Cancer. Urology 1997; 49:65–69.

2 Klotz LH, Goldenberg SL, Jewett MA, Fradet Y, Nam R, Barkin J, Chin J, Chatterjee S: Long-term followup of a randomized trial of 0 versus 3 months of neoadjuvant androgen ablation before radical prostatectomy. J Urol 2003;170:791–794.

3 Boldin MP, Goncharov TM, Goltsev YV, Wallach D: Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 1996;85:803–815.

4 Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM: FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995;81:505–512.

5 Tibbetts MD, Zheng L, Lenardo MJ: The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol 2003;4:404–409.

6 Scaffidi C, Volkland J, Blomberg I, Hoffmann I, Krammer PH, Peter ME: Phosphorylation of FADD/MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. J Immunol 2000;164: 1236–1242.

7 Zhang J, Cado D, Chen A, Kabra NH, Winoto A: Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/MORT1. Nature 1998; 392:296–300.

8 Zhang J, Kabra NH, Cado D, Kang C, Winoto A: FADD-deficient T cells exhibit a disaccord in regulation of the cell cycle machinery. J Biol Chem 2001;276:29815–29818.

9 Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Konishi N: Phosphorylation status of Fas-associated death domain-containing protein (FADD) is associated with prostate cancer progression. J Pathol 2005;206:423–432.

10 Shay JW, Wright WE: Senescence and immortalization: role of telomeres and telomerase. Carcinogenesis 2005;26:867–874.

11 Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Kishi M, Konishi N: Phosphorylation of FADD is critical for sensitivity to anticancer drug-induced apoptosis. Carcinogenesis 2004;25:1089–1097.

12 Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, Lopez-Otin C, Shapiro S, Inada M, Krane S, Allen E, Chung D, Weiss SJ: Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. J Cell Biol 2004;167:769–781.

13 Shimada K, Nakamura M, Ishida E, Higuchi T, Yamamoto H, Tsujikawa K, Konishi N: Prostate cancer antigen-1 contributes to cell survival and invasion though discoidin receptor 1 in human prostate cancer. Cancer Sci 2008;99:39–45.

14 Greider CW, Blackburn EH: Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 1985;43: 405–413.

15 Greider CW, Blackburn EH: A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature 1989;337:331–337.

16 Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, Duncan EL, Reddel RR, Jefferson RA: Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum Mol Genet 1997;6:2011–2019.

17 Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, et al: The RNA component of human telomerase. Science 1995;269: 1236–1241.

18 Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, Arruda I, Robinson MO: A mammalian telomerase-associated protein. Science 1997;275:973–977.

19 Ducrest AL, Szutorisz H, Lingner J, Nabholz M: Regulation of the human telomerase reverse transcriptase gene. Oncogene 2002;21: 541–552.

20 Juo P, Woo MS, Kuo CJ, Signorelli P, Biemann HP, Hannun YA, Blenis J: FADD is required for multiple signaling events downstream of the receptor Fas. Cell Growth Differ 1999;10:797–804.

21 Chen G, Bhojani MS, Heaford AC, Chang DC, Laxman B, Thomas DG, Griffin LB, Yu J, Coppola JM, Giordano TJ, Lin L, Adams D, Orringer MB, Ross BD, Beer DG, Rehemtulla A: Phosphorylated FADD induces NF-κB, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. Proc Natl Acad Sci USA 2005;102:12507–12512.
22 Amit S, Ben-Neriah Y: NF-κB activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach. Semin Cancer Biol 2003;13:15–28.
23 Sheikh MS, Huang Y: The FADD is going nuclear. Cell Cycle 2003;2:346–347.
24 Kabra NH, Kang C, Hsing LC, Zhang J, Winoto A: T cell-specific FADD-deficient mice: FADD is required for early T cell development. Proc Natl Acad Sci USA 2001;98:6307–6312.
25 Gomez-Angelats M, Cidlowski JA: Molecular evidence for the nuclear localization of FADD. Cell Death Differ 2003;10:791–797.
26 Screaton RA, Kiessling S, Sansom OJ, Millar CB, Maddison K, Bird A, Clarke AR, Frisch SM: Fas-associated death domain protein interacts with methyl-CpG binding domain protein 4: a potential link between genome surveillance and apoptosis. Proc Natl Acad Sci USA 2003;100:5211–5216.
27 Shimada K, Nakamura M, Ishida E, Kishi M, Matsuyoshi S, Konishi N: The molecular mechanism of sensitization to Fas-mediated apoptosis by 2-methoxyestradiol in PC3 prostate cancer cells. Mol Carcinog 2004;39:1–9.
28 Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED: Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004;351:1513–1520.
29 Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351:1502–1512.